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In Vitro Chemical and Cellular Tests Applied to Uranium Trioxide with Different Hydration States

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A simple and rapid *in vitro* chemical solubility test applicable to industrial uranium trioxide (UO_3) was developed together with two *in vitro* cellular tests using rat alveolar macrophages maintained either in gas phase or in alginate beads at 37°C. Industrial UO_3 was characterized by particle size, X-ray, and IR spectra, and chemical transformation (e.g., aging and hydration of the dust) was also studied. Solvents used for the *in vitro* chemical solubility study included carbonates, citrates, phosphates, water, Eagle's basal medium, and Gamble's solution (simulated lung fluid), alone, with oxygen, or with superoxide ions. Results, expressed in terms of the half-time of dissolution, according to International Commission on Radiological Protection (ICRP) classification (D,W,Y), varied for different hydration states of UO_3, showing a lower solubility of hydrated UO_3 in solvents compared to basic UO_3 or UO_3 heated at 450 °C. Two *in vitro* cellular tests on cultured rat alveolar macrophages (cells maintained in gas phase and cells immobilized in alginate beads) were used on the same UO_3 samples and generally showed a lower solution transfer rate in the presence of macrophages than in the culture medium alone. The results of *in vitro* chemical and cellular tests were compared, with four main conclusions: a good reproducibility of the three tests in Eagle's basal medium the effect of hydration state on solubility, the classification of UO_3 in terms of ICRP solubility criteria, and the ability of macrophages to decrease uranium solubility in medium.

Introduction

Uranium trioxide ($UO_3 \cdot xH_2O$) is an important intermediate compound in the uranium ore treatment cycle. At the Comurhex factory in Narbonne, France, uranium trioxide is formed by calcining ammonium diuranate (ADU) between 350° and 400°C. Industrial UO_3 obtained in this way is more or less hydrated but may still contain traces of ADU or U_3O_8 (uranium sesquioxide). In dust form, this compound may be inhaled by the worker, and to protect individuals it is essential to know its physicochemical properties and solubility *in vivo*.

To determine the rate of dissolution of compound in the lungs, various *in vitro* chemical and cellular solubility tests have been carried out with alveolar macrophages to categorize UO_3 within the D, W, or Y transferability classes as defined by the International Commission on Radiological Protection (ICRP) (*1*). If the materials are well characterized, *in vitro* tests will enable the problems of dissolution to be investigated and the mechanisms concerned, such as oxidation or complexation, to be elucidated. However, these rapid *in vitro* tests, which use simulated biological liquids, as well as macrophage cultures, must be validated by *in vivo* tests following administration by inhalation.

The results of *in vivo* experiments agree that the transferability of UO_3 is of the D type, as Morrow et al. (*2*) discovered in dogs and Stradling et al. (*3*) in rats. However, the results of *in vitro* tests using different solvents are much more variable. For example, Cooke and Holt (*4*) obtained a class W dissolution in a simulated lung liquid, Pasquier and Bourguignon (*5*) a class D dissolution with a simulated serum, Kalkwarf (*6*) 50% class D, 50% class Y with a Gamble's solution, and Eidson (*7*) a class D behavior. Finally, Stuart et al. (*8*) studied the hydration of UO_3 and its dissolution mechanisms in saline solution and showed that the solubility of UO_3 increased with its hydration state. However, the solubility of UO_3 has yet to be measured using *in vitro* cellular tests.

The aim of the present research is to suggest an *in vitro* methodology that can be used to characterize the physicochemical properties of any sample of UO_3 under investigation. Three *in vitro* tests, one chemical (*9*) and two cellular, were developed to determine the solubility of UO_3 in various solvents or biological environments and to shed light on the dissolution mechanisms. The two *in vitro* cellular tests were carried out using alveolar macrophages from rats, employing two different protocols. In one case, the macrophages were maintained in a gas phase using a method adapted from that described by Voisin et al. (*10*). In the other case, the macrophages were incorporated into alginate beads, using the method of Lirsac et al. (*11*). The UO_3 hydration problem, which results from aging of the dust with the natural humidity of air, is discussed and probably explains the variable results found by the different investigators.

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Materials and Methods

Samples of UO_3

The UO_3 industrial dust from the Comurhex factory in Narbonne (France) takes the form of small agglomerates of various colors measuring between 1 and 10 mm in geometric diameter. The different-colored agglomerates were separated manually and powdered to give a grain size between 1 and 10 μm. Four basic samples were obtained and characterized by X-ray or infrared spectrometry (IR). These samples included the original mixture or industrial UO_3 (A), the orange UO_3 extract (B), the yellow ADU (C) and an oxidized compound UO_3 and U_3O_8 mixture (D).

Examination of aging on samples A and B with air contact showed the production of yellow spots on the orange background of the dust. This led us to prepare additional samples to study the effect of hydration state on solubility: the α and β samples were prepared by heating in a furnace at 37°C at 100% humidity to give a quicker aging or hydration of samples A and B and the γ sample, which corresponds to the B compound dehydrated in an oven at 450°C. The five samples used in the present investigation were A, B, α, β, and γ, details of which are shown in Table 1.

Physicochemical Properties

The characterization of the main physicochemical properties of the five samples, important for understanding *in vitro* tests, involves the following tests.

Particle-Size Distribution. Dust samples, ground to produce particles between 1 and 10 μm in diameter, were aerosolized and the airborne particles sampled with a Andersen Mark II cascade impactor device. The fourth stage of this apparatus corresponded to an activity mass aerodynamic diameter (AMAD) of 3.3 μm.

X-Ray Diffraction. This technique allows the crystalline form of the constituent compounds to be determined. The apparatus used was a Philips PW 1730 spectrometer.

Solid Infrared Analysis. This technique, similar to X-ray diffraction, gives a characteristic absorption spectrum of the vibration bands of the constituent compounds atom groups. Analyses were carried out with a Fourier IR converting spectrometer (1760 X, Perkin Elmer).

In Vitro Chemical Solubility Test

The solubility test used was a static test previously described by Kanapilly et al. (*12*), Kalkwarf (*6*), and Ansoborlo et al. (*9,13*). The solvents used for this dissolution test were distilled water, Eagle's basal medium (BME), Gamble's solution or simulated lung fluid (*9*), carbonates, phosphates, citrates, Gamble + O_2, and Gamble + pyrogallol, superoxide dismutase (SOD), and O_2.

Table 1. Characterization of different UO_3 samples used for *in vitro* tests.

Samples	Method of preparation	X-ray composition
A	Industrial UO_3	UO_3 (90–95%) + ADU (1–5%) + U_3O_8 (1–5%)
B	Extracted UO_3 from A	$UO_3 \cdot xH_2O$ $0.5 < x < 2.5$
α	Industrial UO_3 A hydrated	$A \cdot xH_2O$ $x = 2.5$
β	Extracted UO_3 B hydrated	$UO_3 \cdot xH_2O$ $x = 2.5$
γ	Extracted UO_3 B heated to 450°C	$UO_3 \cdot xH_2O$ $0 < x < 0.5$

Procedures to obtain these solvents have been described by Ansoborlo et al. (*9*).

Each test was conducted over a 20-day period, and the collected samples were analyzed by fluorimetry with a FDTU1 fluorimeter (CEA, France). The results are given as a nondissolved uranium fraction (F) with a time function $F = \Sigma fi \exp(0.693t/Ti)$, where fi is the initial fraction of the i compound and Ti the dissolution half-time. The sum $i = 1$ or 2 was computed using a nonlinear regression program.

In Vitro Cellular Tests

Alveolar Macrophages. The alveolar macrophages (AM) were harvested by bronchoalveolar lavage from rats (OFA strain), anesthetized with pentobarbital, and sacrificied by exsanguination.

Phagocytosis. A suspension of UO_3 (AMAD = 3.3 μm, σ_g = 1.7, concentration = 110 mg/L) was prepared in the cell culture medium and sonicated for 10 min before contact with the cells. The particles were incubated with the macrophages for 1 hr in a culture flask. The adherent cells were washed with a saline solution and either transferred for the assays in gas phase or added to the alginate solution to form beads.

Cell Survival. CELLS IN GAS PHASE. After phagocytosis, the macrophages (1×10^6 cells per plate) were deposited on a Gelman membrane (0.2 μm pore size) and applied to the surface of a reservoir filled with a nutrient medium (*10*). The cells were in direct contact with air that had been saturated with water at 37°C and enriched with 5% CO_2. For sample A, the nutrient medium was medium 199 (Gibco) and for sample B, the nutrient medium was BME (Biomerieux). Both media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, kanamycin, penicillin, and streptomycin. The concentration of uranium was 2 $\mu g/10^6$ cells. Control plates with particles alone (i.e., without cells) were prepared to determine the dissolution rate of UO_3 in the nutrient medium.

CELLS IMMOBILIZED IN BEADS. A suspension of alveolar macrophages that had engulfed the UO_3 (2 $\mu g/10^6$ cells) was added to an alginate solution (2%) to obtain a final concentration of 1.5%. This suspension was then extruded through a succession of catheters with decreasing internal diameters, according to the method described by Lirsac et al. (*11*). The droplets were allowed to fall into Gibco culture medium supplemented with 10% fetal bovine serum and 10 mM calcium, thus forming beads made up of an alginate network. Control beads without macrophages were prepared with the UO_3 suspension (2 $\mu g/10^6$ cells) to test the dissolution rate in the nutrient medium.

Cell Viability. The viability of cells maintained in the gas phase was estimated by measuring the adenosine triphosphate (ATP) content using the method of McEbroy, as modified by Voisin et al. (*10*). The viability of the cells immobilized in beads was estimated by measuring chemiluminescence at 37°C with luminal according to the method of Dyer and Wesleid (*14*).

Twenty-five beads containing a total of 0.5×10^6 macrophages were added to 200 μL of 10^{-3} M luminol. The cells were stimulated by 200 μL of zymosan opsonized with rat serum, and the chemiluminescence was measured during 30 min.

Measurement of Uranium Dissolution. Each day, the nutrient medium contained in the reservoir (AM in gas phase) or in the flask (AM in beads) was replaced, and the amount of solubilized

uranium in the medium was assayed. At the end of the experiment, the uranium concentration remaining on the membranes or in the beads was assayed, making it possible to calculate the total amount of uranium present initially.

Results

Physicochemical Properties

The results of the X-ray diffraction and IR characterization of the UO_3 samples are given in Table 1. Examples of the IR spectra of samples B, β, and γ (corresponding, respectively, to the UO_3 extracted on its own, after hydration, and after drying 1 day in an oven), are given in Figure 1. The change of wavelength of the uranyl peak is due to the hydration rate of UO_3.

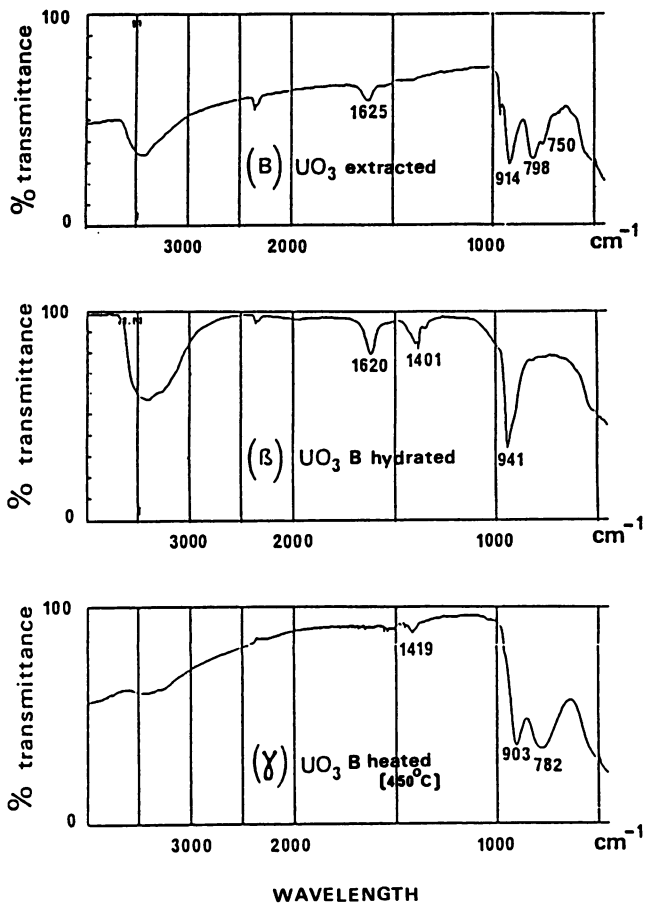

FIGURE 1. Infrared spectra of UO_3 with different hydration states.

In Vitro Chemical Tests

Table 2 shows dissolution results for each of the compounds A, B, α,β, and γ in terms of half-time (*Ti*). The more the UO_3 sample is hydrated, the lower its solubility. The greatest solubility of each compound was obtained with carbonate medium.

In Vitro Cellular Tests

Table 3 shows the results for the dissolution tests of samples A, B, β, and γ in the gas-phase test. The results are shown as percent of uranium solubilized in 24 hr and highlight the effect of hydration on solubility, especially for compound B.

The results for cumulative percentage dissolution of industrial UO_3 (sample A), are shown in Table 4. The results obtained with the gas-phase and alginate-beads protocols are compared. The average quantity of uranium linked to the macrophages after phagocytosis and before the tests was $0.27 \pm 0.11\ \mu g$ ($n = 21$).

The dissolution half-time for sample A in macrophages was 12.2 days for the gas-phase test and 10.8 days for the beads test. The corresponding test reference values were found to be 4.6 and 5.5 days, respectively.

Measurement of ATP viability in alveolar macrophages with UO_3 and in control cells maintained in gas phase is shown in

Table 3. *In vitro* cellular dissolution of UO_3 samples with different hydration states by cultured alveolar macrophages in gas phase (mean percentage ± SD).[a]

% of initial amount dissolved at 24 hr	Sample A	Sample B	Sample β	Sample γ
Uranium	18.3 ± 8.0	38.3 ± 5.0 to 10.8 ± 2.5[b]	10.6 ± 2.0	85.1 ± 3.5
Uranium ± 10^6 alveolar macrophages	6.0 ± 3.6	12.8 ± 3.2 to 5.3 ± 2.9[b]	8.3 ± 1.6	69.7 ± 7.2

[a]Composition of A, B, β, and γ given in Table 1.
[b]Values obtained decrease with increasing time of the hydration state.

Table 4. Industrial UO_3 (sample A) dissolution in gas phase and in beads by cultured cells from rats (mean percentage ± SD).

Elapsed time, days	% Uranium dissolved with macrophages: Test ($n = 21$) in gas phase	Test ($n = 5$) on beads	% Uranium dissolved, reference: Test ($n = 21$) in gas phase	Test ($n = 5$) on beads
1	6.0 ± 3.6	8.9 ± 2.0	18.3 ± 8.0	22.3 ± 9.4
2	9.4 ± 3.8	14.0 ± 3.4	28.8 ± 9.6	ND
3	14.4 ± 5.4	15.0 ± 2.0	33.2 ± 2.8	30.0 ± 3.6
4	22.6 ± 8.9	ND	44.7 ± 1.2	36.7 ± 2.0

ND, not determined.

Table 2. Solubility of different UO_3 samples in several solvents, expressed in term of half-time dissolution.

Samples	*Ti*, days, in H_2O	BME	Gamble	$NaHCO_3$	NaH_2PO_4	Sodium citrate	Gamble + O	Gamble + P + SOD + O_2
(A) Industrial UO_3	2.1	3.6	70	0.8	365	1.2	5	16
(B) Extracted UO_3	4.6	3.8	80	0.6	1286	1.7	2.8	11
(α) Hydrated B	26.4	19.3	125	0.7	2237	2.2	8.2	98
(β) Hydrated B	27.7	16	112	0.5	1708	3.4	7.7	146
(γ)Heated B	1.6	3	60	0.3	507	0.8	0.8	8.3

Abbreviations: *Ti*, dissolution half-time; BME, Eagle's basal medium; Gamble, simulated lung fluid; P, pyrogallol used instead of NADPH to react with O_2 to produce superoxide ions; SOD, superoxide dismutase used to moderate action of superoxide ions.

Figure 2. Figure 3 shows the measurements of chemiluminescence for reference control macrophages and the macrophages with phagocytized UO_3 in the beads test. Results are given for different days (1–4 days).

Discussion

Observation of the hydration state of the extracted UO_3 (sample B) during chemical and cellular tests (Table 3) led to a closer examination of the physicochemical properties of the A, B, α, β, and γ compounds as well as their *in vitro* reactions.

X-ray comparison of compounds A and B (Table 1) shows that these compounds are quite similar, with traces of U_3O_8 and ADU associated with the industrial UO_3. On the other hand, Figure 1 shows the development of a single uranium peak at 940 cm^{-1} for β, whereas B gives two distinct peaks at 914 and 798 cm^{-1}. The IR spectra are the same for compounds A and B. Addition of water molecules, which occurs with aging of dust, induces a chemical transformation. The γ spectrum of compound B heated at 450°C shows the disappearance of the 3430 and 1620 cm^{-1} bands in water and a movement of the two uranium peaks at 903 and 782 cm^{-1}. Thus, hydration and heating change the chemical composition of UO_3.

This physicochemical study demonstrates that the processes involved with UO_3 dust hydration (i.e., industrial A and extracted B) change the crystalline structure by preferential bonding with H_2O.

The results of *in vitro* chemical assays in various media (Table 2) lead to several interesting conclusions:

a) The hydrated dust, i. e., samples α and β, are the least soluble in all media, the exception being $NaHCO_3$, in which solubility is very rapid with a half-time of only about 1 day.

b) Dehydrated UO_3 γ is the most soluble of all the compounds.

c) Carbonates and citrates easily solubilize UO_3 in all its various forms with a class D behavior of between 1 and 3 days. Solubilized uranium in the uranyl forms gives carbonate and citrate soluble complexes as described by Hodge et al. (*15*), which have the compositions $UO_2(CO_3)_3^{-4}$ and $UO_2C_6H_5O_7$, respectively.

d) All compounds of UO_3 are rendered insoluble by phosphates, giving half-times longer than 500 days, i.e., a class Y behavior. Insoluble complexes created, such as $(UO_2)_3(PO_4)_2$ or UO_2HPO_4, with stability constants (K_s) of -49.1 and -10.7, are also described by Hodge et al. (*15*).

e) In Gamble's solution alone, compounds generally have a mixed behavior, between class W and Y, due to the competition between phosphates and carbonates. The phosphatic complexes prevail, however, resulting in a class Y tendency. The addition of O_2 to Gamble's solution gives a class D-type high solubility to the UO_3 due to the accelerated oxidizing of the $UO_3 \cdot xH_2O$ dust in a UO_2^{2+} solution. The action of the superoxide ions in Gamble's solution, due to the addition of pyrogallol with oxygen in the presence of SOD, is more moderate than the action of O_2.

f) The BME or cellular culture medium is richer in proteins than Gamble's solution and gives a reaction of the class-D type with the compounds A, B, and γ, and W type with the hydrated α and β compounds.

g) The natural dust hydration in air atmospheres is different from hydration in water or solvent. Samples A and B, which are orange at the start, quickly turn yellow in water (hydration) and dissolve very quickly. In contrast, samples α and β hydrated in air dissolve more slowly.

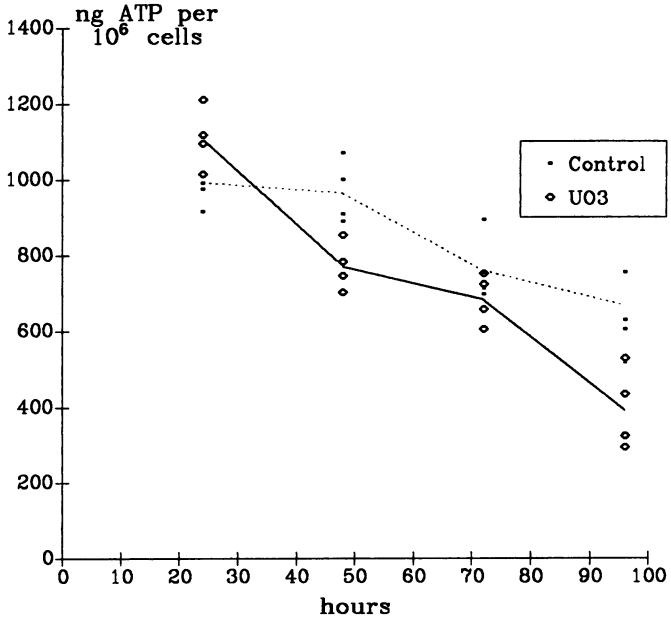

FIGURE 2. Measurement of ATP concentration in pulmonary alveolar macrophages maintained in survival in gas phase. Results given for cells with industrial UO_3 (sample A) and for control cells.

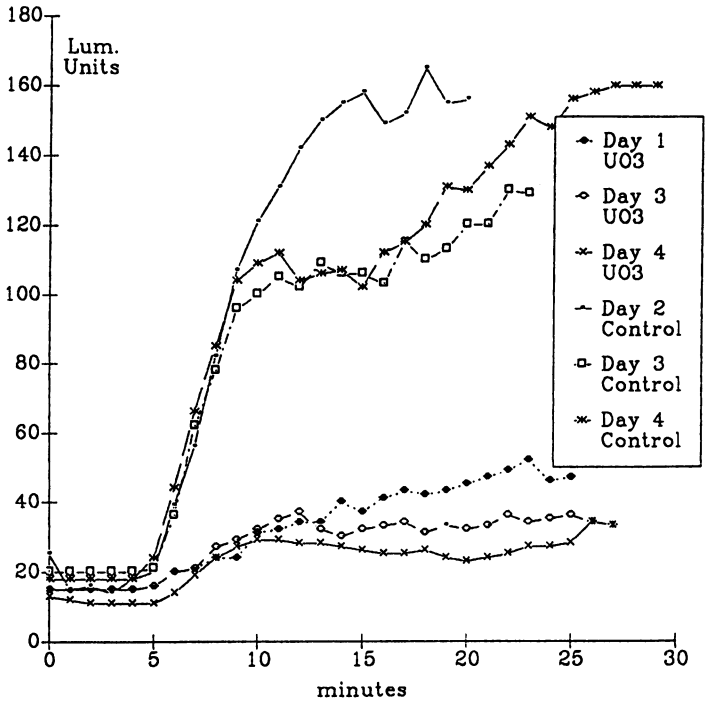

FIGURE 3. Measurement of cellular viability using the chemiluminescence assay on pulmonary alveolar macrophages in the alginate beads test. Results are given for different survival days (1–4).

With both *in vitro* cellular tests applied to the industrial compound A (Table 4), the effect of the macrophages is to reduce solubility. A similar effect can be seen in Table 3 when com-

pounds A, B, β, and γ were tested in gas phase. Variable results with UO_3 compound B were obtained over a 9-month period and have revealed the problem of transformation of the dust due to hydration. Sample B was not stable in time, which explains its decrease in solubility from 38.3% to 12.8%. The observed reduction in solubility may be due to interactions between the dust and constituents of the cellular membrane or to a compound insolubilization by lysosomes. The intracellular precipitation of uranium in the form of uranyl phosphate flakes has already been demonstrated in other cell types by Galle (*16*) and is confirmed by the appearance of insoluble compounds ($t_{½} < 500$ days) when phosphates are used in the *in vitro* test (Table 2). In the lungs, compounds and precipitates are removed by a mucocilary action and eliminated via the gastrointestinal tract.

Decreasing solubility with increasing hydration has been observed (Table 3), confirming the *in vitro* chemical tests results (Table 2). The two types of test gave results that can be compared between themselves and also with the BME *in vitro* chemical test. The gas-phase test, which is much easier to implement, seems suited to research on rapidly soluble compounds. It is difficult to maintain cells in a satisfactory state for more than 5 days, but, with the more complicated test using alginate beads, the macrophages survive longer, providing a useful method for studying less-soluble compounds. When the macrophages are maintained in gas phase, an increasing intracellular ATP concentration (Fig. 2) is sometimes observed after survival for 24 hr; this is probably due to a change in cellular activity related to phagocytosis. For the three tests, the A has a class D solubility with an average half-time of 4-6 days without macrophages and a limit behavior between D and W with macrophages, the average half-time being 11.5 days. Good agrement between the three tests shows that BME is a representative medium for *in vitro* chemical testing.

Conclusion

By using *in vitro* tests on a UO_3 uranium compound with variable hydration levels, we could compare three different techniques for measuring dissolution. Use of these rapid and easily implemented techniques improves the understanding of the mechanisms that underlie the dissolution of a dust in the lungs and enables materials to be classified using ICRP criteria by taking into account their physicochemical characteristics.

The three tests give entirely comparable results and complement one another. The *in vitro* chemical test has the advantage of being simple to use and may assist in understanding solubilization or complexation phenomena in various solvent systems (i.e., carbonates, phosphates, citrates) and may apply to any class D, W, or Y compound. The addition of a macrophage *in vitro* cellular test is an essential complement and reveals insolubilization phenomena due to macrophage action. The gas-phase test is fairly easy to implement and applies more to class D (rapidly soluble) compounds, with which the test may be completed in 4–5 days. The macrophage beads test is more difficult to use but applies to class D and W compounds when the effects of macrophages can be investigated that period of up to 2 weeks.

The main results revealed by this research are *a*) UO^3 compounds are able to hydrate with time, which might explain the discrepancies in the results of measurements of solubility by different authors; *b*) the solubility of UO_3 decreases with increasing hydration in most solvents, as well as the BME for the three tests; *c*) the results for the industrial compound A for the *in vitro* techniques in BME medium (average half-time of 4–6 days) compare well with those in Gamble's synthetic medium + O_2 (half-time of 5 days); and *d*)the distinct insolubilization phenomenon observed in both cellular tests is due to the macrophage action and is reproducible for any hydration level. All these *in vitro* results should be compared to *in vivo* results for each of the given compounds after inhalation by rats.

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